Integrin-mediated Protein Kinase A Activation at the Leading Edge of Migrating Cells

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cAMP-dependent protein kinase A (PKA) is important in processes requiring localized cell protrusion, such as cell migration and axonal path finding. Here, we used a membrane-targeted PKA biosensor to reveal activation of PKA at the leading edge of migrating cells. Previous studies show that PKA activity promotes protrusion and efficient cell migration. In live migrating cells, membrane-associated PKA activity was highest at the leading edge and required ligation of integrins such as $\alpha4\beta1$ or $\alpha5\beta1$ and an intact actin cytoskeleton. $\alpha4$ integrins are type I PKA-specific A-kinase anchoring proteins, and we now find that type I PKA is important for localization of $\alpha4\beta1$ integrin-mediated PKA activation at the leading edge. Accumulation of 3' phosphorylated phosphoinositides [PtdIns(3,4,5)P$_3$] products of phosphatidylinositol 3-kinase (PI3-kinase) is an early event in establishing the directionality of migration; however, polarized PKA activation did not require PI3-kinase activity. Conversely, inhibition of PKA blocked accumulation of a PtdIns(3,4,5)P$_3$-binding protein, the AKT-pleckstrin homology (PH) domain, at the leading edge; hence, PKA is involved in maintaining cell polarity during migration. In sum, we have visualized compartment-specific PKA activation in migrating cells and used it to reveal that adhesion-mediated localized activation of PKA is an early step in directional cell migration.

INTRODUCTION

Directional cell migration is important for processes such as wound healing, leukocyte trafficking, axonal path finding, and cancer cell metastasis. The initial step in most mammalian cell migration is the establishment of cell polarity (Etienne-Manneville and Hall, 2001; Ridley *et al.*, 2003). Once polarity is established, leading edge protrusions, often seen as broad lamellipodia, are pushed forward by actin polymerization and are stabilized by integrin adhesion to the extracellular matrix (ECM) (Ridley *et al.*, 2003). The cell pulls itself along these connections, which provide the traction necessary for migration, while simultaneously dissociating adhesive structures at the cell rear. During migration, localized signaling events lead to the topographically restricted formation and dissolution of attachment structures. Signaling molecules promoting cell protrusion and adhesion accumulate at the front of the cell; these include neuronal Wiskott-

Aldrich syndrome protein, profilin, the Arp 2/3 complex, 3' phosphorylated phosphoinositides [PtdIns(3,4,5)P$_3$], and the Rho family GTPases; Rho, Rac, and Cdc42 (Ridley *et al.*, 2003; Horwitz and Webb, 2003; Pertz *et al.*, 2006). These molecules regulate actin polymerization at the leading edge, which drives membrane protrusion. Thus, understanding the basis for the localization of cellular signaling activities to specific regions of the cell is central to understanding cell migration.

Integrins, transmembrane receptors that mediate the adhesion of migrating cells to the ECM, consist of one α and one β chain whose large extracellular domains form a headpiece that binds to components of the ECM (Ridley *et al.*, 2003). In addition to mediating adhesion, integrins initiate and coordinate the signaling events necessary for cell polarization and motility (Etienne-Manneville and Hall, 2001). $\alpha4$ integrins ($\alpha4\beta1$ and $\alpha4\beta7$) are expressed on highly migratory cells such as leukocytes and neural crest cells (Rose *et al.*, 2002). This class of integrins enhances directional cell migration by the binding of an adaptor, paxillin, to the $\alpha4$ cytoplasmic domain (Liu *et al.*, 1999). Phosphorylation of the $\alpha4$ cytoplasmic domain by c-AMP–dependent protein kinase A (PKA) inhibits paxillin binding and is restricted to the leading edge of broad protrusive lamellipodia and absent from the sides and rear of migrating cells (Goldfinger *et al.*, 2003). This localized phosphorylation of $\alpha4$ at the leading edge is necessary for efficient $\alpha4\beta1$-mediated migration (Goldfinger *et al.*, 2003) because inducing global $\alpha4$ phosphorylation or global blockade of $\alpha4$ phosphorylation both inhibit migration. Furthermore, blocking PKA inhibits protrusion and cell migration

(Goldfinger *et al.*, 2003; Howe *et al.*, 2005; Lim *et al.*, 2007). Thus, PKA-mediated polarized phosphorylation of $\alpha 4$ integrin is necessary for directional cell migration.

The localization of PKA determines its effects on a variety of cellular processes. PKA phosphorylates numerous protein substrates such as ion channels, GTPases, and transcription factors. The substrate specificity of PKA in vivo is controlled by its subcellular compartmentalization and is mediated by interactions of its regulatory subunits with A-kinase anchoring proteins (AKAPs) (for review, see Wong and Scott, 2004). Activation of the cAMP/PKA pathway can induce loss of stress fibers (Dong *et al.*, 1998), activation of Rac and Cdc42 (Feoktistov *et al.*, 2000; O'Connor and Mercurio, 2001), filopodia and lamellipodia formation (O'Connor and Mercurio, 2001; Nishiya *et al.*, 2005), microfilament assembly (Whittard and Akiyama, 2001b), and inhibition of Rho activity (Lang *et al.*, 1996; Ellerbroek *et al.*, 2003), events that occur at the leading edge of migrating cells. PKA-associated AKAPs, such as gravin, WAVE1 and ezrin localize to the leading edge. PKA influences integrin-dependent migration in a variety of cells (for review, see Howe, 2004), and PKA and its phosphorylated substrates are enriched in cell protrusions (Howe *et al.*, 2005; Lim *et al.*, 2007). The presence of AKAPs at the leading edge, the role of PKA in migration, and the localization of $\alpha 4$ phosphorylation to the leading edge suggest that the localized activation of PKA is important in cell migration.

We have developed Förster resonance energy transfer (FRET)-based PKA kinase activity reporters (AKAR1, AKAR2, and AKAR3) that permit the real-time measurement of bulk PKA activity in the cytoplasm of a living cell (Zhang *et al.*, 2001, 2005; Allen *et al.*, 2006). Here, we examine the localization of PKA activity at the plasma membrane of living cells using a membrane-targeted reporter, pmAKAR3 (Allen and Zhang, 2006). We show that PKA is selectively and transiently activated at the leading edge of polarized cells during migration. This localized PKA activity requires the engagement of integrins such as $\alpha 4 \beta 1$ or $\alpha 5 \beta 1$ and polymerized actin. Localized PKA activity does not require the activity of phosphatidylinositol 3-kinase (PI3-kinase), but it is required for the normal polarized accumulation of PtdIns(3,4,5)P$_3$ at the leading edge. Thus, integrins localize PKA activation to the leading edge, and this localized PKA activation is an early event in directional cell migration.

MATERIALS AND METHODS

Plasmids

The following constructs have been described previously: pmAKAR3 (Allen and Zhang, 2006), ICUE2 (Violin *et al.*, 2008), and green fluorescent protein (GFP)-pleckstrin homology (PH)-Akt (Watton and Downward, 1999). To generate pmAKAR3(TA), the *yfp* gene was polymerase chain reaction (PCR) amplified with primers to introduce a 3′ sequence encoding the K-Ras plasma membrane targeting motif KKKKKSKTKCVIM as well as a 5′ SacI site and 3′ EcoRI site. This yellow fluorescent protein (YFP) with the targeting motif replaces the original YFP in the construct AKAR3(TA). pmAKAR3(TA) was then subcloned into a mammalian expression vector derived from pcDNA3. To generate mitoAKB-xx-mcherry constructs, mcherry was PCR amplified to introduce a 5′ SalI site and a 3′ STOP codon and NotI site. The resulting SalI/NotI fragment was subcloned into pCI-mitoAKB-xx-FLAG constructs (Burns-Hamuro *et al.*, 2003), replacing the FLAG epitope with mcherry.

Cell Culture and Monolayer Wounding

Chinese hamster ovary (CHO) cells and CHO cells stably expressing the integrin $\alpha 4$ (CHO-$\alpha 4$) subunit (Goldfinger *et al.*, 2003) were maintained in DMEM complete (supplemented with 10% fetal bovine serum [FBS], 1% nonessential amino acids [Sigma-Aldrich, St. Louis, MO], 50 U of penicillin/ml, and 50 μg of streptomycin sulfate/ml) at 37°C with 6% CO$_2$. Cells were transfected with 2 μg of DNA/60 mm dish using Lipofectamine Plus (Invitrogen, Carlsbad, CA) 24 h before imaging according to the manufacturer's instructions.

Wound assays were performed as described previously (Goldfinger *et al.*, 2003), with minor modifications. Imaging dishes (35 mm) with glass coverslip bottoms were coated overnight at 4°C with either 5 μg/ml CS1 fragment of fibronectin fused to glutathione transferase (GST), which lacks the RGD sequence and specifically engages the $\alpha 4$ integrin as described previously (Jongewaard *et al.*, 1996); or with 5 μg/ml human fibronectin at 4°C and blocked with 1% heat-denatured bovine serum albumin (BSA). Cells were plated and allowed to form a monolayer in imaging media (phenol red-free DMEM, 10% FBS, and 20 mM HEPES, pH 7.4) for 2 h before imaging. A single scratch was made through the monolayer using a 200-μl pipet tip. Cells were washed two times in imaging media to remove cell debris and allowed to migrate for 15 min before live imaging or for 30 min before fixation and immunofluorescence labeling. Function-blocking antibodies directed against integrins $\alpha 4$, HP2/1, or integrin $\alpha 5$, PB1, were applied at 3.0 μg/ml at the time of scratch wounding. The following pharmacological activators or inhibitors were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) and used to treat cells at the following concentrations: forskolin at 25 μM, H89 at 30 μM, wortmannin at 10 μM, Ly294002 at 30 μM, and latrunculin-A at 1 μM.

FRET Imaging

Cells were imaged on a Nikon Eclipse TE-2000 microscope with a 60×/1.4 numerical aperture (NA) oil-immersion objective and a cooled charge-coupled device camera (CoolSNAP HQ; Photometrics, Tucson, AZ) controlled by In Vivo (Media Cybernetics, Bethesda, MD). Simultaneous imaging of both the cyan fluorescent protein (CFP) and YFP emission used a Dual-View (Optical Insights, Tucson, AZ) optical splitter equipped with a 505DCXR (Chroma Technology, Brattleboro, VT) beam splitter. The following excitation filters, altered by a Ludl (Ludl Electronic Products, Hawthorne, NY) wheel changer, were from Semrock (Rochester, NY): 427/10 for CFP, 504/12 for YFP, and 589/15 for mcherry. For imaging of cells expressing pmAKAR3 or ICUE2, the filter set up consisted of the emission filters 464/23 for CFP and 547/31 for YFP paired with FF440/520 multi-edge dichroic beam splitters. For imaging of cells coexpressing pmAKAR3 and mcherry, the set up consists of the emission filters, 464/23 for CFP, 542/27 for YFP and 639/42 for mcherry paired with FF444/521/608 multi-edge dichroic beam splitters. The entire imaging assembly (stage, objective, and sample) were encased in a Plexiglas environmental chamber maintained at 37°C. For prolonged imaging experiments, cells were subhoused in a humidified chamber supplied with 6% CO$_2$. Exposure time was 300 ms, and images were acquired at 2 × 2 binned resolution. Only cells exhibiting comparable expression of pmAKAR3 were selected for imaging and further analysis. Post acquisition analyses were carried out on ImageJ (National Institutes of Health, Bethesda, MD) with the Ratio Plus plugin as follows: CFP and YFP emission images were background corrected by subtracting an averaged value calculated from a cell-free region. The FRET ratio values was calculated by dividing YFP by CFP fluorescence (after background subtraction) and the pseudocolored FRET images generated by viewing the ratiometric data using the Ratio LUT (Look Up Table) plugin running in ImageJ. To maintain the same pseudocolored visual scheme, ratio images for ICUE2 were generated by dividing CFP by YFP fluorescence, because binding of cAMP to this reporter results in loss of FRET.

Immunofluorescence Imaging

Cells were rinsed briefly in Tris-buffered saline (TBS) (50 mM Tris and 150 mM NaCl, pH 7.4) and fixed for 10 min in 3.7% formaldehyde/TBS* (TBS* is 50 mM Tris, 100 mM NaCl, 20 mM Na$_4$P$_2$O$_7$, and 2 mM NaF, pH 7.4). Cells were permeabilized with 0.1% Triton X-100/TBS* for 5 min, blocked with 3% BSA, 2% normal goat serum/TBS* for 1 h, and then incubated with either 10 μg/ml α-PS-$\alpha 4$ (mouse monoclonal antibody clone 633.1; Goldfinger *et al.*, 2003) or 1:150 diluted α-phospho-(S/T) PKA substrate rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA) in block overnight at 4°C. Bound antibodies were detected by the corresponding fluorescein isothiocyanate-conjugated goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and mounted in Prolong Gold (Invitrogen). Widefield immunofluorescence images were acquired on the Nikon TE-2000 microscope with a 60×/1.4 NA oil-immersion objective. Acquired images were processed using ImageJ and Photoshop version 6.0 (Adobe Systems, Mountain View, CA).

Integrin Substrate Micropatterning

The microfluidic chip used to produce the 5-μm-wide CS1 strips on a glass substrate was cast out of Poly(dimethylsiloxane) (PDMS) (Sylgard 184; Dow Corning, Midland, MI) with a lithographically fabricated master mold. The mold was produced by spin-coating a silicon wafer with a 5-μm layer of a UV-curable epoxy (SU8-2005; Microchem, Newton, MA), exposing it to UV-light through a photomask (laser-plotted at a resolution of 50,800 dpi), and removing uncured epoxy. The mold was treated with trimethylchlorosilane (TMCS) to make its surface nonsticky, and then an ~5-mm layer of PDMS prepolymer was poured onto the wafer and cured for 1.5 h at 80°C. The PDMS cast was separated from the mold, cut into individual chips, and inlet and outlet holes were punched using a gauge 20 Luer stub. A complete PDMS chip (not shown) had an array of 300 parallel channels, each 5 μm in width, 5 μm in depth, and 10 mm in length, connected to a common inlet and a common outlet.
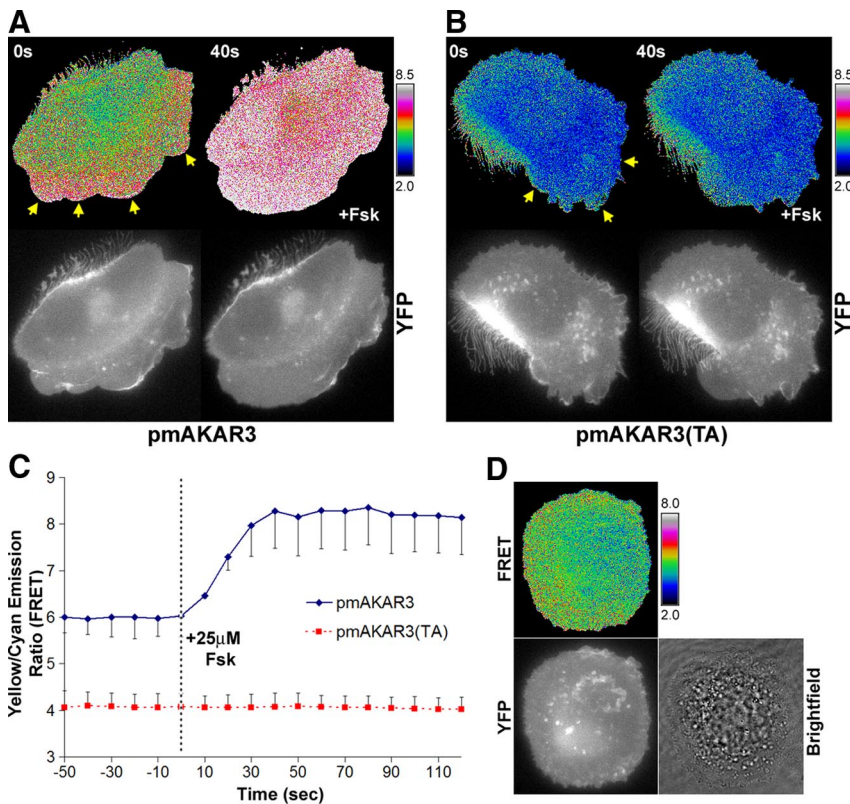
Figure 1. Spatial distribution of PKA activity in polarized cells visualized by pmAKAR3. CHO-$\alpha 4$ cell expressing (A) pmAKAR3 or (B) pmAKAR3(TA) were plated at low densities on the $\alpha 4\beta 1$-specific ligand CS1 and allowed to adhere for 30 min before image acquisition. Pseudocolored FRET images are shown (top), with the corresponding YFP/CFP emission ratio value color-coded scale. The corresponding YFP emission images are shown (bottom) for reference. Yellow arrowheads indicate regions of protrusive behavior visible by time-lapse imaging. Cells were stimulated with 25 μM forskolin at T = 0. (C) FRET ratio values of whole cell area (as represented in A and B) were computed and plotted against the indicated time points. Depicted is the mean and SD of data from three replicate measurements (n = 3 cells). (D) CHO-$\alpha 4$ cell expressing pmAKAR3 were plated on CS1 at a low density for 15 min before imaging. The representative cell as shown was selected based on a lack of a polarized morphology.

To create the pattern of CS1 strips, a PDMS chip was reversibly bonded to a microscope cover glass by overnight baking at 80°C, and a 10 μg/ml solution of CS1-Alexa-Fluor647 (GST-CS1 directly conjugated to Alexa-Fluor647 by using a protein labeling kit (Invitrogen) in phosphate-buffered saline (PBS) was perfused through the microchannel array for ~10 min by applying a differential pressure of 2 psi between the inlet and outlet of the chip (flow rate of ~0.6 μl/min). The microchannels were further perfused with 1% BSA in PBS for 30 min to purge them from the CS1-Alexa-Fluor647 solution and to block the surface of the glass substrate. The PDMS chip was then removed and the cover glass further blocked with 1% BSA in PBS for 0.5 h before seeding cells. Cells were adhered for 1 h before imaging.

Immunoblot Analysis

Western blots for Akt phosphorylation were performed using CHO cells grown to 40% confluence and serum starved (0.5% serum) overnight. Cells were removed with trypsin and rinsed in 2% BSA containing media and held in suspension for 30 min before plating in wells coated with 5 μg/ml fibronectin for the indicated lengths of time in 2% BSA containing media. Blots were first probed with pS473Akt and then stripped and reprobed for total Akt. Both antibodies were purchased from Cell Signaling Technology (9271 and 9272, respectively). LY294002 and H89 were used at concentrations of 50 and 30 μM, respectively, and cells were pretreated with these inhibitors for 1 h before trypsinization and kept in their continued presence for the duration of the experiment.

Western blots were also performed on CHO cells plated overnight in 10% serum after treatment 50 μM forskolin (Fsk). Briefly, cells were pretreated with H89 (30 μM) or control media in the presence of 10% serum for 1 h before the exposure to Fsk for 15 min. Cells were lysed in either control or H89 containing lysis buffer and immediately frozen at −20°C. Phospho-PKA substrates were identified using antibody 9621 from Cell Signaling Technology.

RESULTS

PKA Is Activated Preferentially at the Front of Cells during $\alpha 4\beta 1$ Integrin-mediated Migration

Spatial restriction of integrin $\alpha 4$ phosphorylation by PKA to the leading edge of migrating cells plays an important role in $\alpha 4\beta 1$-dependent cell migration (Goldfinger *et al.*, 2003; Lim *et al.*, 2007). We used a PKA activity biosensor (AKAR) to determine whether localized PKA activity could account

for the spatial patterning of $\alpha 4$ phosphorylation. Initial attempts using AKAR2.2 (Sachs *et al.*, 2007), a soluble cytoplasmic biosensor, prevented visualization of a change in PKA activity at the plasma membrane due to its intense fluorescence in the cytoplasm. Subsequently, we used a compartment-specific reporter targeting the indicator to the plasma membrane by adding a membrane localization sequence from K-Ras, giving rise to plasma membrane-localized pmAKAR2.2 (Sachs *et al.*, 2007). Further improvements resulted in pmAKAR3 (Allen and Zhang, 2006), a third generation plasma membrane localized PKA activity reporter that was used for the studies described here.

To learn whether localized activation of PKA at the leading edge can account for localized $\alpha 4$ phosphorylation, we transfected CHO-$\alpha 4$ cells with pmAKAR3 and plated the cells on CS1, a fragment of fibronectin that serves as a specific ligand for $\alpha 4\beta 1$ (Jongewaard *et al.*, 1996), and time-lapse FRET micrographs of the cells were taken. A cell that was randomly polarized exhibited increased FRET emission ratio at the membrane protrusions concurrent with the formation of lamellipodial protrusion (Figure 1A, arrows, and Supplemental Movie 1). Addition of 25 μM forskolin, an agonist for adenylate cyclase, resulted in increased FRET ratio throughout the cell with a saturated maximal response after 40 s (Figure 1, A and C, and Supplemental Movie 1). As a control for the specificity of the biosensor probe, CHO-$\alpha 4$ cells expressing pmAKAR3(TA), containing a threonine-to-alanine point mutation in the PKA substrate region and thus rendering it resistant to PKA phosphorylation (Allen and Zhang, 2006), exhibited low overall FRET, even within membrane protrusions (Figure 1B, arrows) and stimulation with forskolin did not affect the FRET ratio (Figure 1, B and C), establishing that the forskolin-induced increase in FRET requires phosphorylation of the biosensor's substrate domain.
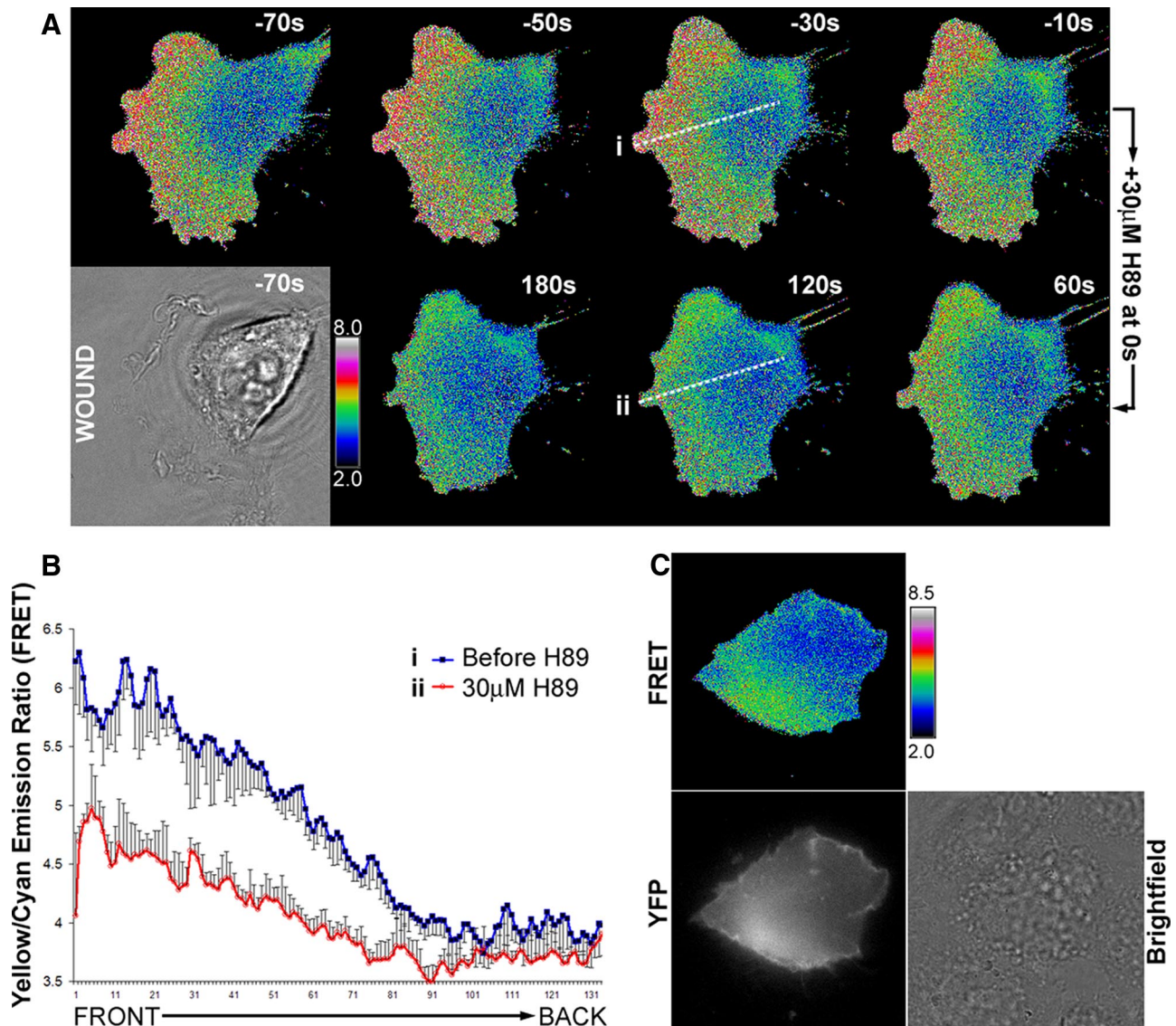
Figure 2. PKA activity gradient in cells migrating into a scratch-induced wound. (A) CS1-adhered monolayers of CHO-α4 cells expressing pmAKAR3 were wounded by scratching and imaged 15 min later by time-lapse microscopy. Images were acquired before and after the addition of 30 μM H89 at T = 0. (B) Plots of the line profile scans for the cell shown in A, with the front-to-back cell orientation depicted on the x-axis. As shown are the averaged FRET ratio values for five successive image frames (5-s intervals) centered at -30 s (i) and 120 s (ii) as shown in A. Error bars are the standard deviations. (C) Representative FRET image of a cell embedded within the monolayer located two to three rows behind the wound edge.

Whereas high FRET ratio values, indicative of increased PKA activity, were consistently observed in the lamellipodial protrusions of polarized cells (Figure 1A), nonpolarized cells did not exhibit elevated FRET ratios at the cell periphery relative to the central areas of the cell (Figure 1D). Thus, membrane-localized PKA activity, as indicated by the plasma membrane anchored FRET-based reporter pmAKAR3, is increased in protrusions of polarized cells.

We scratch-wounded CHO-α4 cell monolayers plated on CS1 to achieve robust directional cell migration (Goldfinger *et al.*, 2003; Lim *et al.*, 2007). Along the margin of the wound, the cells rapidly polarized and extended a broad advancing lamellipodium toward the wound as they began to migrate (Supplemental Movie 2). Similar to the randomly protruding cells shown in Figure 1A, the leading edge of migrating CHO-α4 cells exhibited increased FRET that was sustained during protrusion, indicative of an enrichment of PKA activity (Figure 2A and Supplemental Movie 3). A line profile analysis of FRET emission ratios within the individual migrating cell indicated a gradient of PKA activity that was highest at the leading edge tapering gradually to basal levels at the middle and back end of the cell (Figure 2B). A quantitative analysis of FRET ratios in three zones, corresponding to the front, middle, and rear of polarized cells, revealed that the differences in PKA activity between the front and back were statistically significant (Supplemental Figure S1). We consistently observed leading edge enrichment of PKA activity in all cells that exhibited persistent protrusive behavior indicative of active migration (n > 50 cells). Furthermore, leading edge FRET was sustained for the duration of protrusive activity that was observed to persist for up to 6 min (data not shown). Addition of 30 μM H89, an inhibitor of

PKA, to the actively migrating cells resulted in dramatic loss of FRET activity at the leading edge membranes within 60 s, indicating that the FRET signal depended on continuing PKA activity and confirming that the signal from the pmAKAR3 probe is rapidly reversible. As expected from previous studies (Goldfinger *et al.*, 2003; Howe *et al.*, 2005; Lim *et al.*, 2007), inhibition of PKA activity also resulted in reduced protrusive activity (Figure 2A and Supplemental Movie 3) and cessation of cell migration. A line profile analysis for the same region 60 s after H89 addition exhibited a loss of the front-to-back PKA activity gradient (Figure 2B).

Because pmAKAR3 is a rapidly reversible reporter of PKA activity, the present data suggest that increased PKA activity is continuously present at the leading edge. Furthermore, treatment of cells with H89 before wounding prevented the increased FRET ratios at the wound edge (data not shown). Addition of H89 to actively migrating cells led to reduction of protrusive activity and loss of the gradient of FRET ratios, indicating that the spatial polarization of FRET was reflective of PKA activity. Furthermore, the increased FRET at the leading edge was not due to a path length effect or registration artifact because it was not observed in cells expressing pmAKAR3(TA), the probe that lacks the PKA phosphorylation site (Supplemental Figure S2). Cells located within the monolayer several rows away from the wound edge (Figure 2C and Supplemental Figure S3) were not polarized and did not exhibit the high levels of localized PKA activity observed for cells at the wound edge. Thus, only the polarized cells migrating out from the wound edge manifested increased PKA activity in the leading edge. Furthermore, blocking PKA markedly suppressed cell protrusion and migration, confirming the importance of this kinase in cell migration.

Integrins Mediate the Activation of PKA at the Leading Edge of Migrating Cells

Previous studies show that $\beta 1$ integrin-mediated adhesion can activate PKA (Kim *et al.*, 2000; Meyer *et al.*, 2000; Plopper *et al.*, 2000; Whittard and Akiyama, 2001a; Howe *et al.*, 2005; Lim *et al.*, 2007). Because migrating cells are continuously forming new integrin-mediated contacts at the leading edge (Ridley *et al.*, 2003), we examined the role of integrin ligation in polarized PKA activation. CHO-$\alpha 4$ cells plated on the $\alpha 4\beta 1$-specific ligand CS1 were scratch wounded and allowed to migrate in the presence of either HP2/1 or PB1, function-blocking antibodies for integrins $\alpha 4\beta 1$ and $\alpha 5\beta 1$, respectively. When $\alpha 4$ integrins were blocked by treatment with HP2/1, cells at the wound edge failed to migrate and exhibited little to no enrichment of PKA activity at the leading edge (Figure 3A, top, and Supplemental Figure S4). In contrast, blockade of $\alpha 5\beta 1$ with PB1 neither blocked migration nor reduced FRET in leading edge protrusions (Figure 3A, bottom). Thus, $\alpha 4$ integrins mediate the activation of PKA at the leading edge of migrating cells.

The foregoing experiments showed that $\alpha 4\beta 1$-mediated cell migration was associated with activation of PKA at the leading edge of migrating cells. To assess whether this phenomenon was $\alpha 4$-specific, we studied the migratory behavior of CHO cells plated on fibronectin. Because these cells do not express $\alpha 4\beta 1$, they adhere and migrate on fibronectin by using primarily integrin $\alpha 5\beta 1$ (Goldfinger *et al.*, 2003). When a monolayer of CHO cells plated on fibronectin was wounded, an increase in the FRET ratio occurred along the leading edge (data not shown). This phenomenon was sustained when CHO cells were allowed to migrate in the presence of HP2/1 antibody (Figure 3B, top), indicating that treatment with HP2/1 did not result in nonspecific
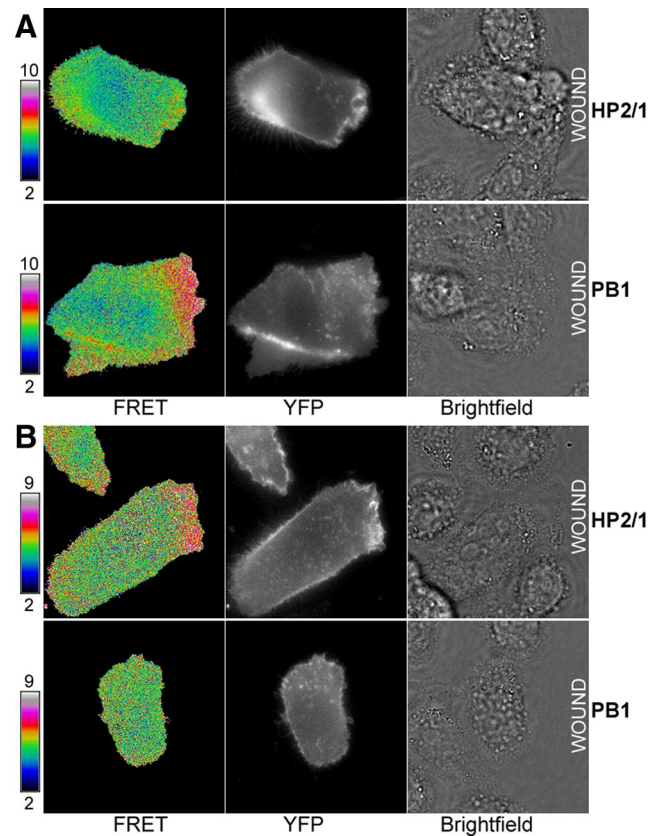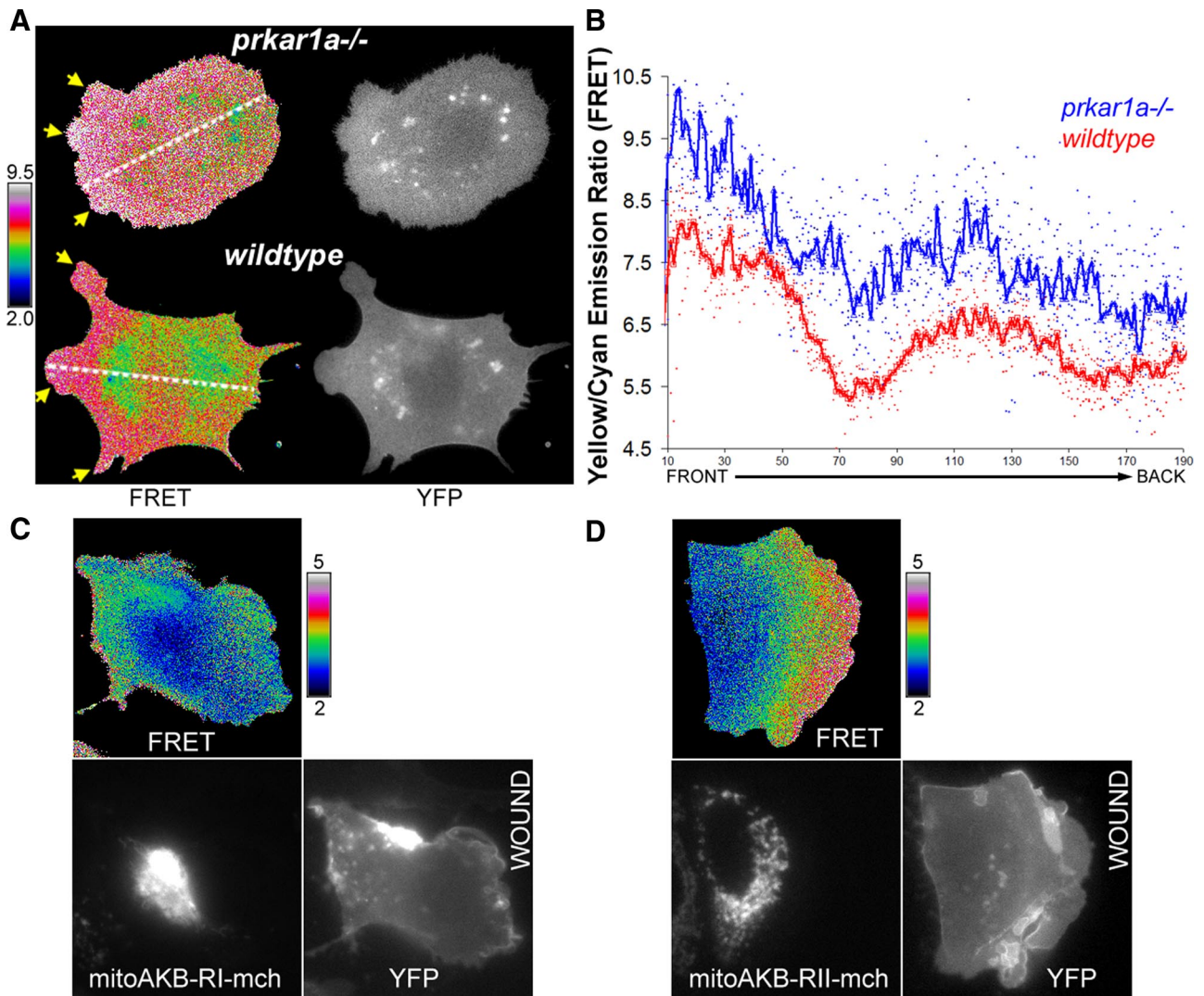

Figure 3. Activation of PKA at the leading edge is integrin dependent. (A) CHO-$\alpha 4$ cell monolayers adhered on CS1 and expressing pmAKAR3 were wounded, and migration was allowed to proceed in the presence of 2 μg/ml HP2/1 (anti-$\alpha 4$) or PB1 (anti-$\alpha 5$) monoclonal antibodies as indicated. (B) CHO cell monolayers adhered on fibronectin and expressing pmAKAR3 were wounded, and migration was allowed to proceed in the presence of 2 μg/ml HP2/1 or PB1 monoclonal antibodies as indicated.

inhibition of PKA activity or migration. In contrast, blockade of integrin $\alpha 5\beta 1$ with PB1 inhibited the migration of CHO cells on fibronectin and blocked the increased PKA activity (Figure 3B, bottom, and Supplemental Figure S5) at the leading edge. Thus, multiple integrins can mediate the activation of PKA at the leading edge of migrating cells.

Type I PKA-specific Modulation of PKA Activity at the Leading Edge

The cytoplasmic domain of integrin $\alpha 4$ functions as an AKAP to tether type I PKA at the plasma membrane (Lim *et al.*, 2007). To investigate the role of type I PKA in PKA activation at the leading edge, mouse embryonic fibroblasts (MEFs) expressing $\alpha 4$ integrins and pmAKAR3 were adhered on CS1 and assayed by FRET ratio imaging. Wild-type MEFs extend multiple protrusions that exhibited high levels of PKA activity (Figure 4, A and B). In sharp contrast, loss of the type Iα PKA regulatory subunit (PKA-RIα) in *prkar1a*−/− MEFs led to increased PKA activity observed throughout the cell (Figure 4, A and B). This was not unexpected, because loss of PKA-RIα leads to increased basal PKA activity as a result of excess free PKA catalytic subunits (Amieux *et al.*, 2002; Lim *et al.*, 2007). This deregulation of PKA activity could complicate interpreta-

Figure 4. Integrin $\alpha4\beta1$-specific activation of PKA at the leading edge is type I PKA dependent. (A) Wild-type or *prkar1a*$-/-$ mouse embryonic fibroblasts stably expressing $\alpha4\beta1$ integrins were transfected with pmAKAR3, adhered on CS1, and imaged as isolated cells. Yellow arrowheads indicate regions of cell protrusion. (B) Profile plots shown are the averaged ratiometric values for six parallel line scans centered around the white line tracing as seen in A, with the front-to-back cell orientation indicated on the *x*-axis. (C) CHO-$\alpha4$ cells doubly transfected with mcherry-mitoAKB-RI (C) or mcherry-mitoAKB-RII (D) and pmAKAR3 were adhered as monolayers on CS1 and migration was induced by wounding. The mitoAKB constructs express type I or type II specific PKA binding peptides that sequester the relevant PKA isoform to the mitochondria.

tions of the role of PKA-RIα in spatial control of PKA activity. Consequently, we used mislocalization of type I PKA as an alternative strategy to examine its role in polarized PKA activation. We used a mitochondrial-targeted type I-specific AKAP peptide, mitoAKB-RI, to sequester type I but not type II PKA on mitochondria (Burns-Hamuro *et al.*, 2003; Lim *et al.*, 2007). Expression of mitoAKB-RI in CHO-$\alpha4$ cells (Lim *et al.*, 2007), resulted in marked reduction of PKA activity at the leading edge of migrating cells (Figure 4C). In contrast, expression of mitoAKB-RII, which specifically sequesters type II PKA to the mitochondria, had no discernible effect on enrichment of leading edge PKA activity in CHO-$\alpha4$ cells migrating on CS1 substrate (Figure 4D). This shows that in cells undergoing integrin $\alpha4\beta1$-dependent migration, type I PKA is largely responsible for the localized PKA activity at the leading edge membrane.

Role of F-Actin and Cellular Protrusion in PKA Activation at the Leading Edge

The foregoing studies showed that global blockade of integrin engagement prevented the activation of PKA at the leading edge of migrating cells. Because high-affinity integrins are preferentially localized to the leading edge (Kiosses *et al.*, 2001), this is a site at which many integrins are newly engaging ligands. To directly assess the role of local engagement of integrins in the localized PKA activation, we used a micropatterned matrix substratum of alternating 5-μm-wide strips of CS1 and uncoated surface (Figure 5A). The pattern was formed by reversibly bonding to a microfluidic chip made of PDMS with an array of parallel 5-μm-wide microchannels, 5-μm partitions between them, and perfusing through the microchannels a solution of CS-1. When CHO-$\alpha4$ cells were seeded on this micropatterned substrate, increased FRET was observed
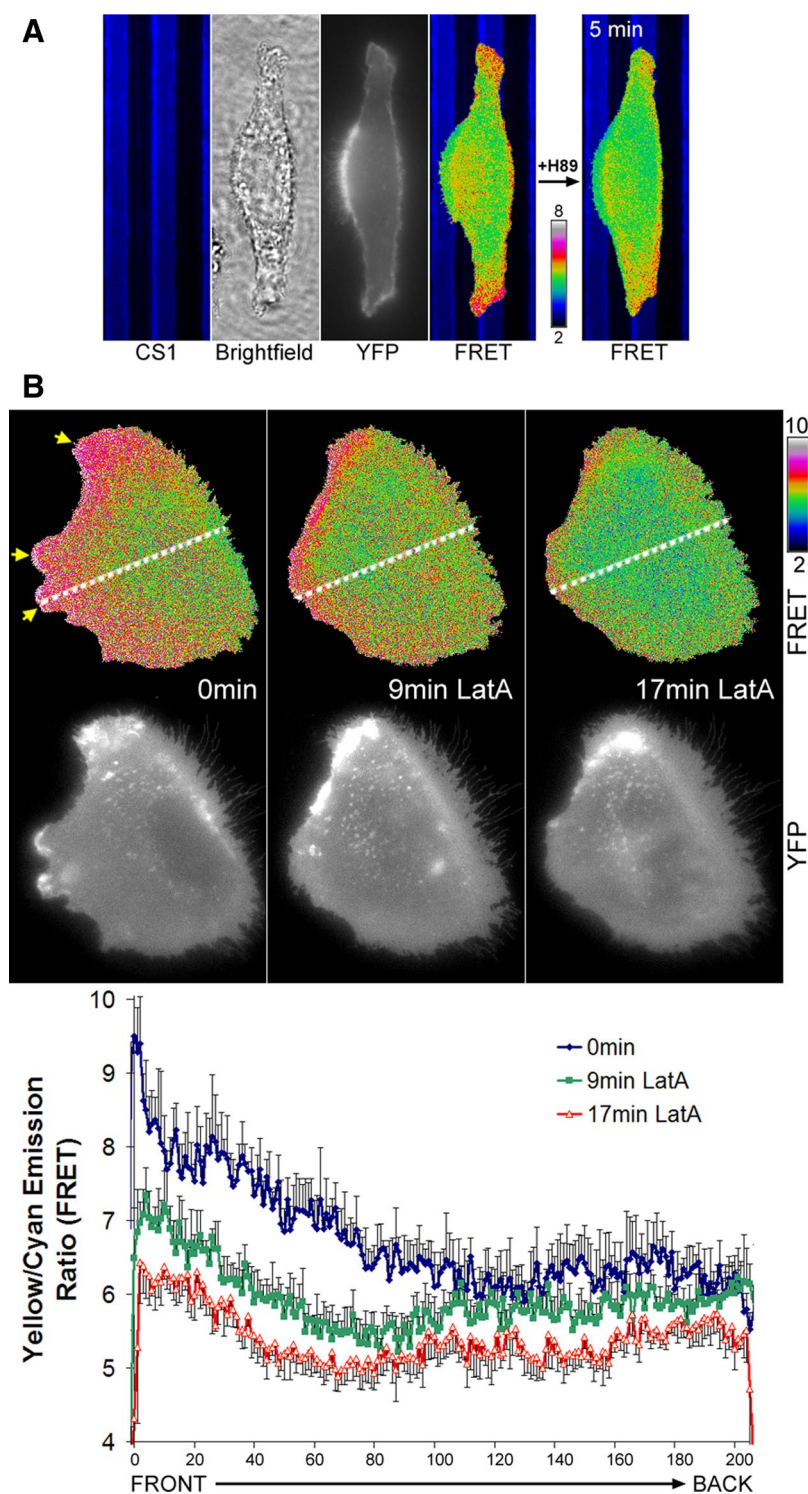
Figure 5. Enrichment of PKA activity occurs at cellular protrusions and requires actin polymerization. (A) A microfluidic chamber was used to coat a glass coverslip with Alexa647-labeled CS1 substrate in 5-μm-wide strips (blue) interspersed with uncoated glass surface (black in panel 1). Shown is a representative CHO-α4 cell expressing pmAKAR3 adhered on the micropatterned substrate and extending protrusions along the CS1 coated surface. The pseudo-colored FRET images as shown were overlaid upon the image of the coating to facilitate its alignment relative to the cell. (B) An isolated adhered pmAKAR3-transfected CHO-α4 cell was treated with 1 μM latrunculin-A at T = 0 and imaged for FRET. Yellow arrowheads indicate regions of cell protrusion visible in time-lapse image sequence. Profile plots shown are the averaged ratiometric values for six parallel line scans centered around the white line tracing.

primarily where the cells made contact with the adhesive substrate (Figure 5A and Supplemental Figure S6) and was absent from regions that made contact with CS1 ligand-free zones. This increased FRET activity was blocked by addition of H89 (Figures 1D and 5A). Thus, both antibody inhibition experiments (Figure 3) and cell adhesion to micropatterned substrates indicate that integrin engagement by ligands is required for increased membrane-associated PKA in cell protrusions.

The micropatterned substrates also revealed a close association between regions of the cells that were protruding and increased PKA activity (Figure 5A). The association of protrusions with increased PKA activity raised the possibility that actin polymerization, the principal biochemical mechanism that drives protrusion, might play a role in maintaining this adhesion-dependent PKA activity. To test this idea, we treated CHO-α4 cells adherent to CS1 with latrunculin-A. After addition of latrunculin-A, the greatest decline in PKA activity was
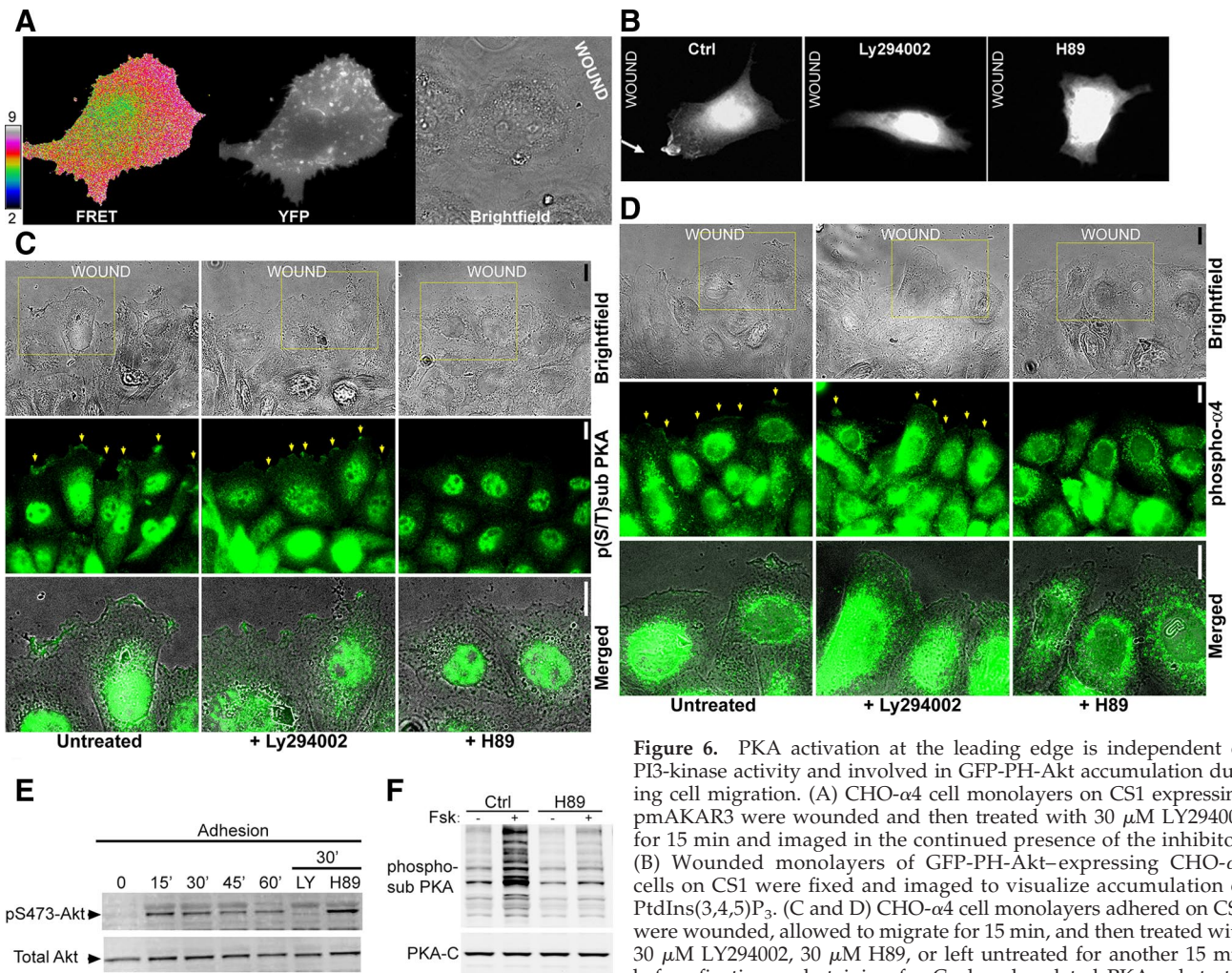
Figure 6. PKA activation at the leading edge is independent of PI3-kinase activity and involved in GFP-PH-Akt accumulation during cell migration. (A) CHO-α4 cell monolayers on CS1 expressing pmAKAR3 were wounded and then treated with 30 μM LY294002 for 15 min and imaged in the continued presence of the inhibitor. (B) Wounded monolayers of GFP-PH-Akt–expressing CHO-α4 cells on CS1 were fixed and imaged to visualize accumulation of PtdIns(3,4,5)P$_3$. (C and D) CHO-α4 cell monolayers adhered on CS1 were wounded, allowed to migrate for 15 min, and then treated with 30 μM LY294002, 30 μM H89, or left untreated for another 15 min before fixation and staining for C phosphorylated PKA substrates [p(S/T)substrate PKA] and (D) phosphorylated α4 (phospho-α4) by using the appropriate antibodies. Bottom, magnified and merged images of the regions bounded by yellow rectangles. (E) CHO-α4 cells were adhered to fibronectin-coated wells for the indicated times, lysed, fractionated by SDS-PAGE, and immunoblotted to detect Akt phosphorylation at serine 473 (pS473-Akt). The blot was reprobed with antibody recognizing total Akt protein. LY, LY294002. (F) To ensure the efficacy of H89 inhibition of PKA, cells were treated with 50 μM Fsk in the presence or absence of H89 and immunoblotted with phospho-PKA substrate antibody.

observed at the leading edges and protrusive structures (Figure 5B). Consistent with loss of new actin polymerization, protrusive activity ceased in the presence of latrunculin-A. This decline in PKA activity in protrusions occurred in spite of the fact that the latrunculin-A–treated cell maintained a polarized morphology. Indeed, PKA activity in protrusions decreased to the low level seen at the lateral and central compartments of the cell by 17 min after latrunculin-A addition (Figure 5B). Thus, increased PKA activity at the membrane protrusions is sustained by actin polymerization.

PKA Activation at the Leading Edge Does Not Require PI3-Kinase Activity

The foregoing results indicate that the increase in PKA at the leading edge is specific for protruding areas, implying that PKA activation might be the result of forward protrusion during migration. The localized accumulation of PtdIns(3,4,5)P$_3$ by activated PI3-kinase at the leading edge has been implicated as an early step in the polarization and forward protrusion of cells migrating in chemoattractant gradients (Wang *et al.*, 2002; Funamoto *et al.*, 2002; Huang *et al.*, 2003).

This raises the possibility that active PI3-kinase at the leading edge is necessary for the polarized activation of PKA. Surprisingly, although inhibition of PI3-kinase with LY294002 altered lamellipodial dynamics and reduced the migration of α4β1-expressing CHO cells into a wound, it did not block PKA activation at the leading edge (Figure 6A and Supplemental Figure S7). In the majority of cells treated with LY294002, PKA FRET seemed elevated at both the front, sides, and rear, demonstrating the ability of PKA to become activated in the absence of PI3K activity with some disruption of normal polarity. As a control for the efficacy of LY294002 treatment, we examined the subcellular localization of GFP-PH-Akt, which functions as a biosensor for PI3-kinase activity because the PH domain of Akt is recruited by PtdIns(3,4,5)P$_3$ (Meili *et al.*, 1999; Servant *et al.*, 2000). After wounding, ~60% of cells exhibited increased fluorescence of GFP-PH-Akt at the leading edge in untreated cells, whereas all cells treated with LY294002 exhibited no accumulation of GFP-PH-Akt to the leading edge (Figure 6B). Furthermore, we also observed no accumulation of GFP-PH-Akt to the leading edge in cells treated with H89

(Figure 6B). To further confirm the observation that PKA activation was independent of PI3-kinase activity, we examined the phosphorylation of PKA substrates at the leading edge by staining fixed cells with phospho-PKA substrate-specific antibodies, as an indicator of PKA activity. Leading edge staining for phospho-PKA substrates was observed in LY294002-treated CHO-α4 cells migrating on CS1 (Figure 6C). In contrast, treatment with H89 abolished leading edge staining for PKA-phosphorylated substrates. When we stained cells to detect phosphorylated α4 integrins, a similar result was obtained in that LY294002 did not inhibit leading edge accumulation of phosphorylated α4, whereas H89 did so (Figure 6D). Identical results were obtained for CHO-α4 cells treated with wortmannin to inhibit PI3-kinase (data not shown).

We next examined the requirement of PKA activity for the accumulation of PtdIns(3,4,5)P$_3$ by measuring phosphorylation and activation of Akt (PKB) (Burgering and Coffer, 1995; Franke *et al.*, 1995), a kinase regulated by PtdIns(3,4,5)P$_3$. Adhesion of cells induced phosphorylation of Akt in a time-dependent manner (Figure 6E). The adhesion-mediated stimulation of phosphorylated Akt was not blocked by H89 treatment (Figure 6E), although the same concentration of H89 prevented PKA phosphorylation of substrate proteins (Figure 6F). Together, the data suggests that PKA activation at the leading edge is independent of PI3-kinase activity and in contrast to the accumulation of GFP-PH-Akt at the leading edge, activation of Akt, a reflection of global levels of PtdIns(3,4,5)P$_3$, does not require PKA activity. Thus, blocking PKA activation prevents the accumulation of GFP-PH-Akt in protrusions but does not block activation of a PtdIns(3,4,5)P$_3$-dependent enzyme.

Polarized Cells Exhibit a Front-to-Back Gradient of cAMP

The foregoing studies established that PKA activation at the leading edge of migrating cells is dependent on integrins and the integrity of the actin cytoskeleton but not on PI3-kinase activity; however, these results did not explain why the PKA activity is markedly increased at the leading edge. Because PKA is activated by cAMP, we hypothesized that increased cAMP might be generated at the front of migrating cells. To address this possibility, we transfected CHO-α4 cells with a cytoplasmic FRET-based biosensor for cAMP termed ICUE2 (DiPilato *et al.*, 2004; Violin *et al.*, 2008). These cells were attached to CS1 substrate and imaged as they underwent migration into a wound. In a majority of migrating cells, a linear front-to-back cAMP gradient was observed (Figure 7). Thus, we conclude that the localization of increased cAMP activity can partially account for the enrichment of PKA activity in protrusions during integrin-mediated cell migration.

DISCUSSION

Leading edge PKA Activity in Cell Migration
Cell migration requires the precise localization of cellular signaling events. Here, we use a membrane-targeted reporter of PKA activity and show that transient PKA activation is localized to the leading edge of migrating cells. Furthermore, integrin ligation is necessary, but not sufficient, for the localized activation of PKA. This effect was not specific for an α integrin subunit type, because it was mediated by either α4β1 or α5β1 integrins. However, leading edge PKA activity is mediated primarily by type I PKA for cells undergoing α4β1-dependent migration. High levels of

localized PKA activation at leading edge protrusions require polymerized actin as evidenced by loss of leading edge FRET upon latrunculin-A treatment. Leading edge PKA activity was not abolished in cells treated with PI3-kinase inhibitors; on the contrary, PKA FRET increased in the lateral and back compartments of the cell. Our data also establish a mechanism for localized PKA activity at the cell leading edge because migrating cells exhibit a front-to-back decreasing gradient of cAMP. These results establish that integrin-dependent activation of PKA at the leading edge of cells is independent of PI3-kinase activity and plays a role in maintenance of cell polarity and directional cell migration.

The specificity of PKA signaling is achieved by its compartment-specific tethering mediated by AKAPs. The advent of the FRET-based PKA activity biosensor (Zhang *et al.*, 2001), coupled with compartment specific targeting (Allen and Zhang, 2006), afforded us the opportunity to observe PKA signaling in specific compartments in living cells. The specific targeting of pmAKAR3 to the plasma membrane both enhanced the resolution of AKAR FRET by eliminating cytoplasmic background, and allowed for analysis of PKA activity in the vicinity of membrane-associated substrates. Previous work showed preferentially phosphorylation of PKA substrates at the front of migrating cells; our studies now show that this is due to increased PKA activity and is an integrin-dependent process. For example, PKA can phosphorylate proteins that are at or near the leading edge including VASP (Butt *et al.*, 1994; Howe *et al.*, 2002; Howe *et al.*, 2005), LIM and SH3 domain protein-1 (Chew *et al.*, 2000), Rho GTPase (Dong *et al.*, 1998), and c-Src (Abrahamsen *et al.*, 2003). Our finding that PKA is activated at the leading edge suggests a role for this localized activation in the preferential phosphorylation of these substrates here. By using FRET and real-time imaging in moving cells, we see that PKA activity is sustained during the forward migration of the cell. Furthermore, changes in global PKA activity can modulate signaling enzymes such as Rac (O'Connor and Mercurio, 2001) or Cdc42 (Feoktistov *et al.*, 2000). Cdc42-mediated filopodia and Rac-mediated lamellipodia are the usual morphological consequences of forward protrusion. PKA contributes to Rac activation in growing pseudopodia (Howe *et al.*, 2005), and we had previously found that restricted α4 integrin phosphorylation contributes to the selective activation of Rac at the front of migrating cells (Nishiya *et al.*, 2005). The present results show that PKA activity is localized to the leading edge, accounting for restricted α4 phosphorylation and Rac activation. Furthermore, these results are consistent with the idea that PKA substrates, which can contribute to forward protrusion by regulating Rac and CDC42, are selectively phosphorylated at the leading edge (Howe *et al.*, 2005; Lim *et al.*, 2007).

We find that integrin ligation is necessary for the localized activation of PKA at the leading edge; however, this effect is not specific for a particular alpha subunit. PKA activation was evident at the leading edge in cells migrating via either α4β1 or α5β1 integrins. Activation of PKA upon α5β1-mediated adhesion is necessary for appropriate regulation of calcium channels in smooth muscle cells (Lopez *et al.*, 1987). Furthermore, adhesion mediated by β1 integrins leads to an increase in intracellular cAMP (Plopper *et al.*, 2000; Whittard and Akiyama, 2001a), transient PKA-mediated phosphorylation of vasodilator-stimulated phosphoprotein (Howe *et al.*, 2002), and an associated increase in polymerized F-actin (Whittard and Akiyama, 2001b). Interestingly, cell detachment (Howe and Juliano, 2000) and mechanical strain on β1 integrins (Meyer *et al.*, 2000) can activate PKA. Cells are continuously forming new integrin-mediated attachments at
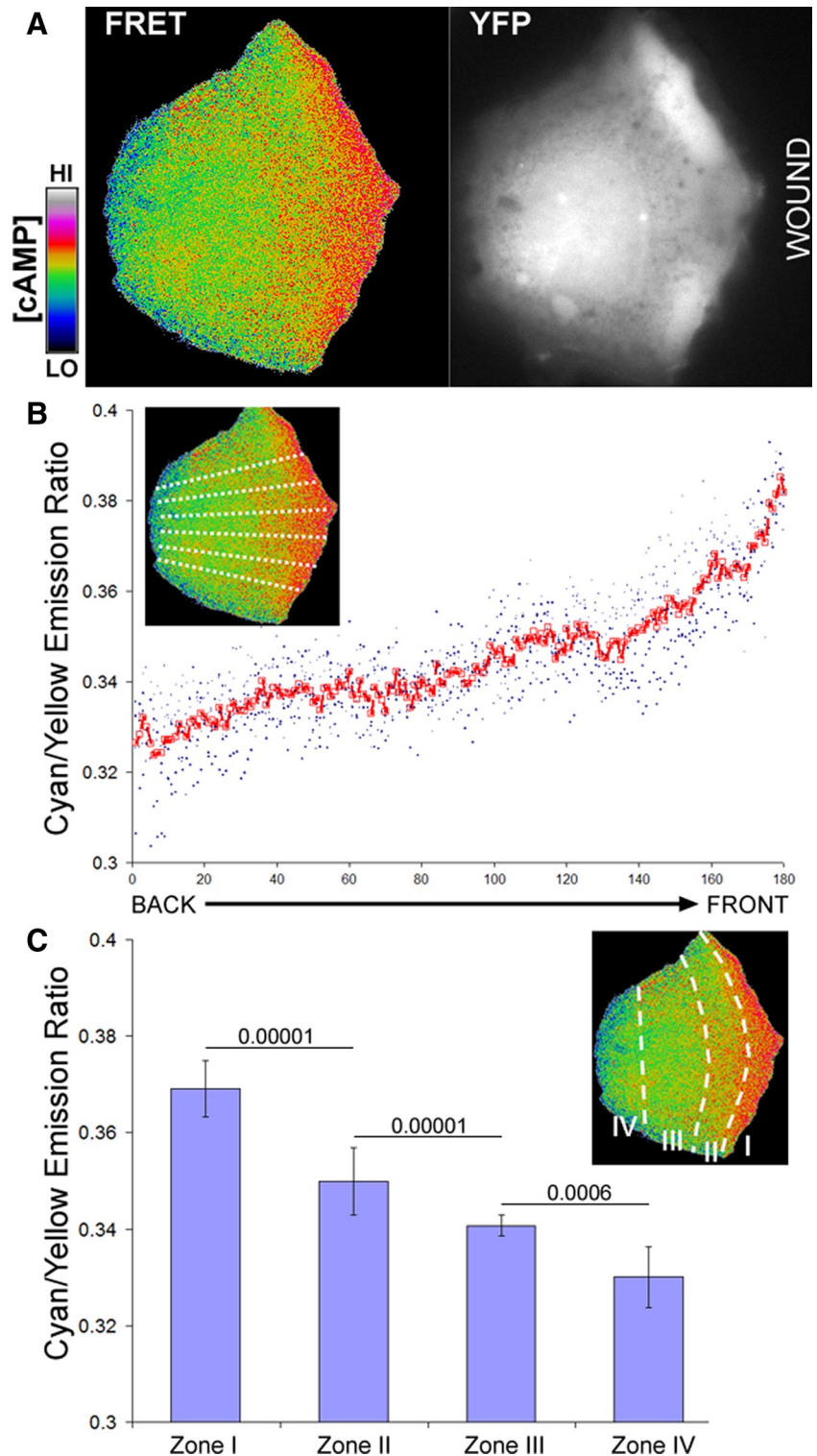
Figure 7. Polarized cells exhibit a front to back cAMP gradient. (A) CHO-α4 cells expressing ICUE2, a FRET-based cAMP binding biosensor, were grown as monolayers on CS1, were wounded, and imaged as described above. The pseudocolor coding scheme as shown is for CFP divided by YFP emission ratio to reflect the fact that this reporter exhibits loss of FRET upon cAMP binding. (B) Plots of the line profile scans for the cell shown in A, with the front-to-back cell orientation depicted on the x-axis. Profile plot is the averaged ratiometric values and data distribution for six line scans as shown in the inset. (C) The ratiometric image was subdivided into four zones as shown in the inset and ratio values randomly sampled within these zones (n = 5 per zone). Error bars are standard deviations and p values, using the Student's paired t test, are shown for the indicated comparisons.

the cell anterior and exerting force on those attachments (Ridley *et al.*, 2003). Meyer *et al.* (2000) found that G_s proteins mediated this form of integrin-mediated mechanotransduction. Combined with our data, these studies suggest that forces exerted on integrin attachments at the leading edge activate PKA by stimulating cAMP synthesis through G_s-mediated activation of adenylate cyclase. The abundance of AKAPs at the leading edge, and our finding that localized PKA activation is an important early determinant of cell polarity, underscores the need for further analysis of the relative roles of AKAPs and local cAMP synthesis in the localized activation of PKA.

The α4 integrins are strongly promigratory and have an unusual relationship with PKA signaling. They bind directly to type I PKA (Lim *et al.*, 2007) and are phosphorylated by PKA at the leading edge to promote $\alpha4\beta1$-dependent migra-

tion. This direct relationship suggests that type I PKA might be of particular significance for PKA activation when cells migrate via α4 integrins. In support of this idea, we found that sequestration to the mitochondria of type I, but not type II PKA, resulted in reduced leading edge PKA activity, complementing and explaining previous studies (Lim *et al.*, 2007) that identified the selective importance of type I PKA in α4 phosphorylation and α4β1-dependent migration (Lim *et al.*, 2007). The fact that type II PKA has been implicated in directional migration in fibroblasts and smooth muscle cells (Howe *et al.*, 2005), and in growth cone motility (Han *et al.*, 2007), indicates other AKAPs are able to couple PKA activation to migration. Indeed, in contrast to α4β1-dependent migration, we find that α5β1-dependent migration and PKA activation at the leading edge is inhibited by sequestration of type I PKA, and to a lesser extent by type II PKA (data not shown). Similarly, Howe *et al.* (2005) found that HT31, a peptide that is a preferential inhibitor of type II PKA–AKAP interactions, blocked preferential phosphorylation of PKA substrates in pseudopodia. Together, these data suggest that localized PKA activation is important in migration; however, different integrins use differing mechanisms of tethering PKA to effect this localized activation.

We found that cell migration was associated with a gradient of increased cAMP toward the leading edge. The work of Ingber and colleagues (Meyer *et al.*, 2000) elegantly demonstrated that G protein-dependent cAMP synthesis requires both ligation of integrins and mechanical strain transmitted through the cytoskeletal network. We observed little spatial enrichment of FRET in nonmigrating, nonpolarized cells, indicating that integrin ligation alone is not sufficient for activating PKA. Elevated PKA FRET was consistently seen in sites of active protrusions and required polymerized actin, suggesting that force generated by motile cells exerted upon integrin adhesions constitutes one mechanism to sustain cAMP synthesis. Thus, we propose that cAMP gradients resulting from integrin ligation in association with protrusive forces contribute to the localized PKA activation at the leading edge.

Localized PKA Activity Is Involved in Leading Edge Localization of PtdIns(3,4,5)P₃

The PI3-kinase product PtdIns(3,4,5)P$_3$ accumulates preferentially at the leading edge of some polarized cells (Wang *et al.*, 2002) and is required for polarization and migration of certain cells in response to chemotactic gradients (Funamoto *et al.*, 2002; Wang *et al.*, 2002; Huang *et al.*, 2003; Schneider and Haugh, 2005). PtdIns(3,4,5)P$_3$ can positively regulate fundamental components of the migration machinery, such as Rac activation (for review, see Welch *et al.*, 2003). In our experiments, PI3-kinase inhibition altered lamellipodial behavior and partially inhibited migration; yet, it failed to block the polarized activation of PKA or phosphorylation of its substrates. On the contrary, PI3-kinase inhibition increased PKA activity at the lateral sides and back of the cell. These results suggest that the integrin-dependent activation of PKA at the leading edge is independent of PI3-kinase; however, PI3-kinase activity is important for maintaining polarized PKA signaling. It is possible that PI3-kinase is necessary for maintaining proper cAMP gradients in cells, because cardiomyocytes lacking PI3-kinase γ have increased cAMP levels (Kerfant *et al.*, 2005), resulting from loss of phosphodiesterase activity (Kerfant *et al.*, 2007). Spatial restriction of PKA and PI3-kinase activity may act together to maintain polarity and promote directional cell migration.

Surprisingly, PKA activity was necessary for leading edge accumulation of GFP-PH-Akt at the cell front. PKA inhibition did not inhibit Akt phosphorylation, which is dependent on PI3-kinase–mediated synthesis of PtdIns(3,4,5)P$_3$. Although PKA is not required for global accumulation of PtdIns (3,4,5)P$_3$, it seems to control the bulk quantity of the PtdIns(3,4,5)P$_3$ at the leading edge as reported by GFP-PH-Akt. In *Dictyostelium* and human neutrophils, the localization of PtdIns(3,4,5)P$_3$ during migration is specified by the localization and activation of enzymes that synthesize the lipid, e.g., PI3-kinases, and those that degrade it, e.g., phosphatase with sequence homology to tensin (PTEN) (Funamoto *et al.*, 2002; Wang *et al.*, 2002; Li *et al.*, 2005). Our finding that PKA is upstream of the accumulation of GFP-PH-Akt in migrating cells points the way to studies to analyze the role of PKA in the regional regulation of PI3-kinases and PTEN. Our finding that integrin-mediated localized PKA activation can control polarized protrusion suggests that the pathway elucidated here will be widely used in directional cell migration.

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